INFECTION AND IMMUNITY, Nov. 1994, p. 5177–5182
0019-9567/94/$04.00+0

Vol. 62, No. 11

Nitric Oxide Is Involved in Control of *Trypanosoma cruzi*-Induced Parasitemia and Directly Kills the Parasite In Vitro

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Received 23 May 1994/Returned for modification 6 July 1994/Accepted 23 August 1994

This study was carried out to determine the role of reactive nitrogen intermediates in *Trypanosoma cruzi* infection. In vitro, splenocytes obtained during the acute phase of infection produced elevated amounts of nitric oxide (NO) that were correlated with the resistance or susceptibility of the animals. In vivo, the levels of NO_2^- plus NO_3^- in plasma during the later phase of infection were higher in C57BL/6 mice than in BALBL/c mice. The treatment of infected C57BL/6 mice with inhibitors of NO synthase increased parasitemia and mortality. Finally, we found that the NO donor drug *S*-nitroso-acetyl-penicillamine is able to kill trypomastigotes in vitro in the absence of any other cells, suggesting a direct NO-mediated killing of *T. cruzi*.

Trypanosoma cruzi, the causative agent of Chagas' disease, is a hemoflagellate protozoan parasite that infects humans and a wide variety of mammals. Although the infected host develops specific cellular and antibody-mediated immune responses, the chronic infection is maintained by a few trypomastigotes in the blood and gives rise to a major public health problem, namely, the transmission of Chagas' disease through blood transfusions (36).

Following the infection of normal mice with *T. cruzi*, the parasites are able to survive and multiply in nucleated cells. However, in immune mice the parasites are extensively destroyed by macrophages (40). The importance of these cells in the resistance to infection has been demonstrated in vivo (31) and in vitro (28, 30, 31) and with experiments using agents that alter macrophage function, such as colloidal thorium dioxide and silica (12). Gamma interferon (IFN-γ) plays a central role in inducing the activation of macrophages which results in the inhibition of *T. cruzi* intracellular replication (28, 30, 38). The antitrypanosome activity of activated macrophages is thought to be mediated by the generation of hydrogen peroxide (28, 32). However, the amount of hydrogen peroxide released by macrophages during the infection of susceptible mice is higher than that observed in the resistant mice (34). Furthermore, the treatment of activated macrophages with phorbol myristate acetate to exhaust their respiratory burst or with catalase, superoxide dismutase, or sodium benzoate to scavenge respiratory burst metabolites fails to inhibit their ability to kill *T. cruzi* in vitro (25). In addition, a cell line with a defective respiratory burst has been shown to be able to kill the parasites upon activation with both IFN-γ and lipopolysaccharide (10).

It is now generally accepted that nitric oxide (NO) or related nitrogen oxides produced by activated macrophages are cytostatic or cytotoxic for a variety of pathogens, including *Leishmania major* (17, 23, 24), *Mycobacterium bovis* (9), *Toxoplasma gondii* (1), *Schistosoma mansoni* (21), *Cryptococcus neoformans* (13), *Trypanosoma musculi* (41), and *T. cruzi* (10). NO is generated from the terminal guanidino nitrogen atom of L-arginine by an NADP-dependent enzyme, NO synthase (20). In macrophages, the enzyme activity is inducible by cytokines such as IFN-γ (5) and tumor necrosis factor alpha (TNF-α) (5, 6). TNF-α acts in an autocrine fashion to amplify the actual synthesis and release of NO by IFN-γ-primed macrophages (16, 22). Activated macrophages do not synthesize NO or display antimicrobial activity in the presence of N^G-monomethyl-L-arginine (L-NMMA) (14), N^ω-nitro-L-arginine (NO-ARG) (19), or L-imino-ethyl-L-ornithine (L-NIO) (26), substrates that inhibit nitrogen oxidation of L-arginine.

Although the microbicidal activity of NO in vitro has been well established, its role in mediating host resistance in vivo to obligate intracellular parasites requires further investigation. In *L. major* infection in mice, it was suggested that the treatment of resistant animals with NO synthase inhibitors promoted an increase in the size of the parasite-induced lesion (7, 24). These results suggest that the reactive nitrogen intermediate is a major effector molecule in the inhibition of intracellular proliferation of *L. major*. The present investigation was undertaken to assess the role of NO in the control of *T. cruzi* infection in resistant mice and to evaluate its trypanocidal activity in vitro. First, we address the question of whether NO production during the infection correlates with the resistance or susceptibility of the animals.

C57BL/6 and BALB/c mice were infected with the Y strain of *T. cruzi*, and the parasitemia, serum nitrate, and levels of nitrite produced by the splenocytes cultured for 48 h were determined. Female BALB/c and C57BL/6 mice (6 to 8 weeks old) were infected intraperitoneally with 10^4 blood-derived trypomastigote forms of the Y strain of *T. cruzi*. Parasitemia levels were evaluated in 5 μl of blood obtained from the tail vein. Spleen cells from normal or *T. cruzi*-infected BALB/c and C57BL /6 mice were washed in Hanks' medium and incubated for 4 min with a lysing buffer (9 parts of 0.16 M ammonium chloride and 1 part of 0.17 M Tris). The erythrocyte-free cells were then washed three times and suspended to 5×10^6 cells per ml in RPMI 1640 (Flow Laboratories, Inc., McLean, Va.) supplemented with 5% fetal calf serum (Hyclone, Logan, Utah), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. One milliliter of the cell suspension was added to each well of 24-well tissue culture plates and incubated for 48 h at 37°C in a humidified, 5% CO_2 incubator. Subsequently, the supernatants were harvested, filtered through 0.22-μm-pore-size membranes, and stored at −20°C until used for the nitrate assay. The nitrite concentration in the culture supernatants or in serum was assayed in a microplate by mixing 0.1 ml

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of culture supernatant or serum with 0.1 ml of Griess reagent (15). The A_{550} was read 10 min later, and the NO_2^- concentration was determined by reference to a standard curve of 1 to 100 μM $NaNO_2$. Serum samples from infected resistant (C57BL/6) and susceptible (BALB/c) mice were collected from the retro-orbital plexus on the days after infection indicated in the figures. Nitrate was reduced to nitrite with nitrate reductase as described elsewhere (35), and the nitrite concentration was determined by the Griess method.

The parasitemia had similar patterns in both strains, peaking at day 8 and decreasing thereafter, although its level was higher in BALB/c than in C57BL/6 mice (Fig. 1a). The mortality rate was 80% in BALB/c mice, whereas none of the C57BL/6 mice died during the infection. NO_2^- levels were significantly increased from days 4 to 13 and 6 to 15 in the supernatants of splenocytes from *T. cruzi*-infected C57BL/6 and BALB/c mice, respectively. During the first 8 days of infection, the concentrations of NO_2^- secreted by splenocytes from resistant mice were significantly higher and were detectable earlier than those observed in susceptible mice (Fig. 1b), suggesting a possible involvement of NO in the control of the disease in the former animals. When the animals began to control the parasitemia on the 9th day after infection, NO_2^- production was similar in both groups and decreased thereafter to control levels by day 15. The addition of 10 μg of trypomastigote lysate to the splenocyte cultures did not significantly change NO_2^- production (data not shown). Other investigators using different parasite strains observed that peritoneal or spleen cells from BALB/c mice infected with *T. cruzi* released NO when incubated without further stimuli, with no major difference in the production of NO species by mice infected with virulent or mildly virulent parasite strains (29).

In addition, we determined the levels of NO_2^- plus NO_3^- in the serum of mice at different days after *T. cruzi* infection. The concentrations of NO_2^- plus NO_3^- in serum in infected mice increased beginning 8 days after infection. On days 11, 13, and 15, the concentrations of NO_2^- plus NO_3^- were significantly higher in C57BL/6 mice than in BALB/c mice (Fig. 1c). While the levels of NO_2^- plus NO_3^- in the plasma of BALB/c mice decreased, becoming similar to those of noninfected mice by day 15, the concentration in resistant mice remained high until the end of the experiment (15 days after infection). The production of NO in other susceptible and resistant mice strains is currently under investigation. The kinetics of NO production detected in spleen cell supernatants (Fig. 1b) and in serum (Fig. 1c) were not similar. This apparent discrepancy could be explained by the fact that the concentration of NO_3^- in serum is a consequence of NO produced by different tissues, which may present a different time course of NO synthase induction, as demonstrated in mice infected with *Corynebacterium parvum* (33).

In an effort to understand the role of NO in the control of infection in vivo, we treated infected C57BL/6 mice daily with inhibitors of NO synthase to inhibit the increase in, but not eliminate the baseline production of, NO. The animals received daily intraperitoneal injections of saline with or without L-NMMA or NOARG (Sigma) diluted in saline (50 mg/kg of body weight) during the first 15 days of infection. The first dose was given 4 h after infection. Parasitemia and mortality were determined during the acute phase of infection. Treatment of infected mice with L-NMMA and NOARG reduced the levels of NO_2^- and NO_3^- in serum by 62 and 53% at day 8 of infection, respectively. Treatment during the first 15 days of infection resulted in significant increases in the levels of parasitemia on days 8 and 9 compared with levels in infected mice receiving only saline. On day 8, the increase in parasitemia was fivefold higher than that in nontreated animals (Fig. 2a). In addition, treated mice showed a higher mortality. While the control resistant mice did not die during the infection, the L-NMMA- or NOARG-treated groups showed 100% mortality by days 27 and 22 after infection, respectively

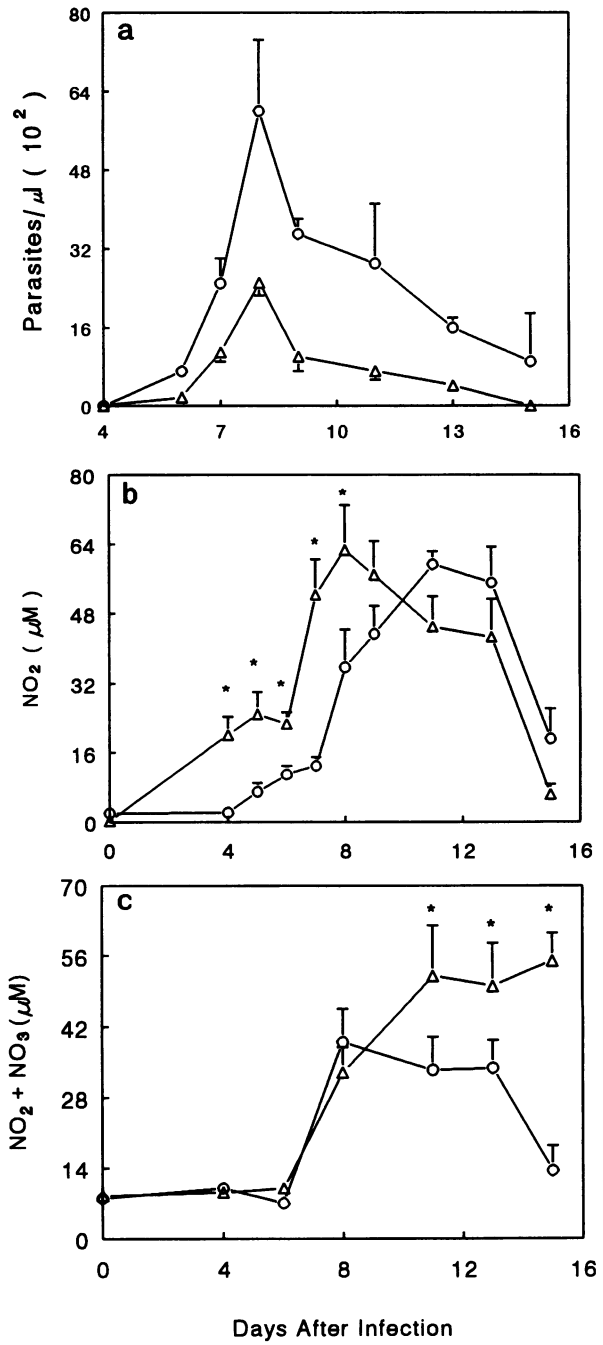

FIG. 1. Kinetics of parasitemia and NO production of BALB/c (circles) and C57BL/6 (triangles) mice during *T. cruzi* infection. Parasitemia levels (a), nitrite concentrations in the culture supernatants of splenocytes (b), and concentrations of nitrite plus nitrate in serum (c) were determined at the days indicated. Each point (mean ± standard error of the mean) corresponds to five mice in an experiment representative of three similar experiments. *, $P > 0.05$ (Mann-Whitney test) compared with infected BALB/c mice.

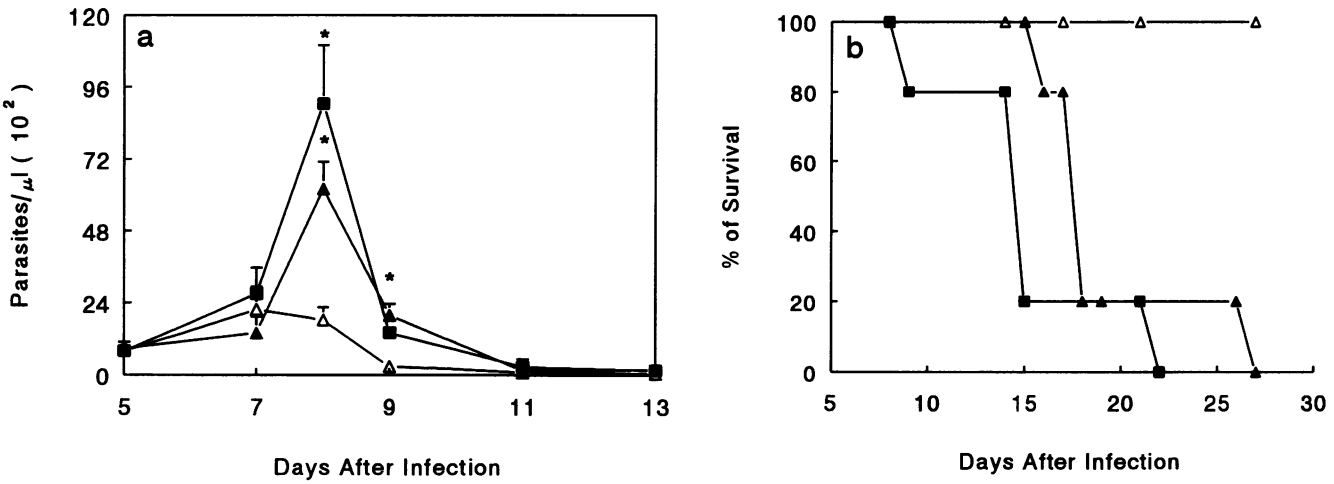

FIG. 2. NO synthase inhibition increases the parasitemia and mortality of *T. cruzi*-resistant mice. C57BL/6 mice were infected with 10^4 *T. cruzi* trypomastigotes and treated with saline (open triangles), L-NMMA (closed triangles), or NOARG (squares) at a dose of 50 mg/kg 4 h later. The animals were treated daily until day 15 after infection. The levels of parasitemia (a) were determined on the indicated days and were significantly different from the saline (control) group on days 8 and 9 after infection (*, $P < 0.05$; Kruskal-Wallis test). Percent survival (b) of each group ($n = 5$) was determined on the days indicated and is representative of two independent experiments.

(Fig. 2b). Although there is evidence that L-NMMA is more potent than NOARG in blocking NO synthesis by macrophages in vitro (19), in our in vivo experiments the effects of both drugs on the *T. cruzi*-induced parasitemia and mortality were similar. As previously described (27), the dose of NO synthase inhibitors used was not toxic to the animals, since the treatment of normal mice did not change their weights or their survival rate. In the infected L-NMMA-treated C57BL/6 mice weight loss was observed 2 to 3 days before death but was not different from the weight loss observed in untreated infected BALB/c mice (data not shown). These results provide evidence for the participation of NO in the resistance to *T. cruzi* infection observed in C57BL/6 mice. The involvement of NO in the resistance to *L. major* in different strains of mice has also recently been demonstrated (7, 24).

Since we demonstrated above that treatment of infected mice with inhibitors of NO synthase exacerbated the infection by *T. cruzi* in vivo, we also investigated whether IFN-γ-induced macrophage trypanocidal activity is dependent on intermediates of L-arginine metabolism. For the in vitro experiments, trypomastigotes and amastigotes were grown in and purified from rat myoblast cells (L6E9). Peritoneal macrophages were harvested from mice injected 3 days previously with 1 ml of 3% (wt/vol) sodium thioglycolate (Difco). The cells (5×10^5/ml) were plated onto 96- or 24-well plates and were infected at a parasite/cell ratio of 4:1 for 90 min. Extracellular parasites were removed with six washes of RPMI 1640, and the cells were incubated at 37°C in 5% CO_2 for the times indicated in the legend to Fig. 3 in the presence of 100 U of recombinant murine IFN-γ (5.2×10^6 U/ml; Genentech Inc., San Francisco, Calif.) and/or 200 μM L-NIO (kindly provided by S. Moncada, The Wellcome Research Laboratories, Beckenham, United Kingdom). The growth of parasites in macrophages was evaluated by counting the trypomastigotes released at various times after infection and by counting the intracellular amastigote forms as described previously (32). Macrophage monolayers were infected with *T. cruzi* following activation with IFN-γ in the absence or presence of L-NIO. Incubation of the macrophages with 100 U of IFN-γ almost completely inhibited the intracellular replication of *T. cruzi* (Fig. 3). The incubation of infected cells with L-NIO (200 μM) had no effect on the course of infection. On the other hand, when macrophages were incubated with IFN-γ plus L-NIO, there was a dramatic reduction in the inhibitory effects of IFN-γ on *T. cruzi* intracellular replication. Similar results were obtained by counting the trypomastigotes released from infected cells (Fig. 3a) or by determining the number of intracellular amastigotes 48 h after infection (Fig. 3b). In vitro, it has been demonstrated that the trypanocidal activity of a macrophage cell line (J774-G8) and of human macrophages activated with IFN-γ and TNF-α could be mediated by a NO-dependent mechanism since it was partially inhibited by L-NMMA (10). Here, we have also shown that the trypanocidal activity of IFN-γ-activated macrophages was completely blocked by L-NIO, a more potent specific inhibitor of NO synthase in macrophages (Fig. 3). In the presence of L-NIO, the production of NO by IFN-γ-stimulated macrophages was completely attenuated (data not shown). The NO synthase inhibitors NOARG and L-NMMA were also able to inhibit the IFN-γ-induced trypanocidal activity but to a lesser extent than was observed for L-NIO (data not shown).

Having shown that the trypanocidal activity of IFN-γ-activated macrophages could be mediated by a product of L-arginine metabolism, we examined the trypanocidal activity of the NO donor *S*-nitroso-acetyl-penicillamine (SNAP; kindly provided by S. Moncada) in a macrophage-free system. *T. cruzi* trypomastigotes grown in L6E9 fibroblasts were suspended in RPMI 1640 at a dilution of 2.0×10^6 parasites per ml, and 100-μl aliquots of this suspension were plated onto 96-well plates. One hundred microliters of SNAP or control penicillamine (Sigma) diluted in RPMI 1640 to the concentration indicated in Fig. 4 was added to the parasites and incubated at 37°C in 5% CO_2 for 12, 24, or 48 h. Parasite viability was subsequently assayed by determining the number of motile forms in a hemocytometer. The SNAP doses used did not have a toxic effect on macrophages or *Escherichia coli* in vitro (3a). The addition of SNAP to the trypomastigotes resulted in a dose- and time-dependent killing of the parasites. Twelve hours after the incubation of *T. cruzi* trypomastigotes with 250 or 500 μM SNAP, the number of viable parasites was reduced by 33 and 37%, respectively. At lower doses, killing was

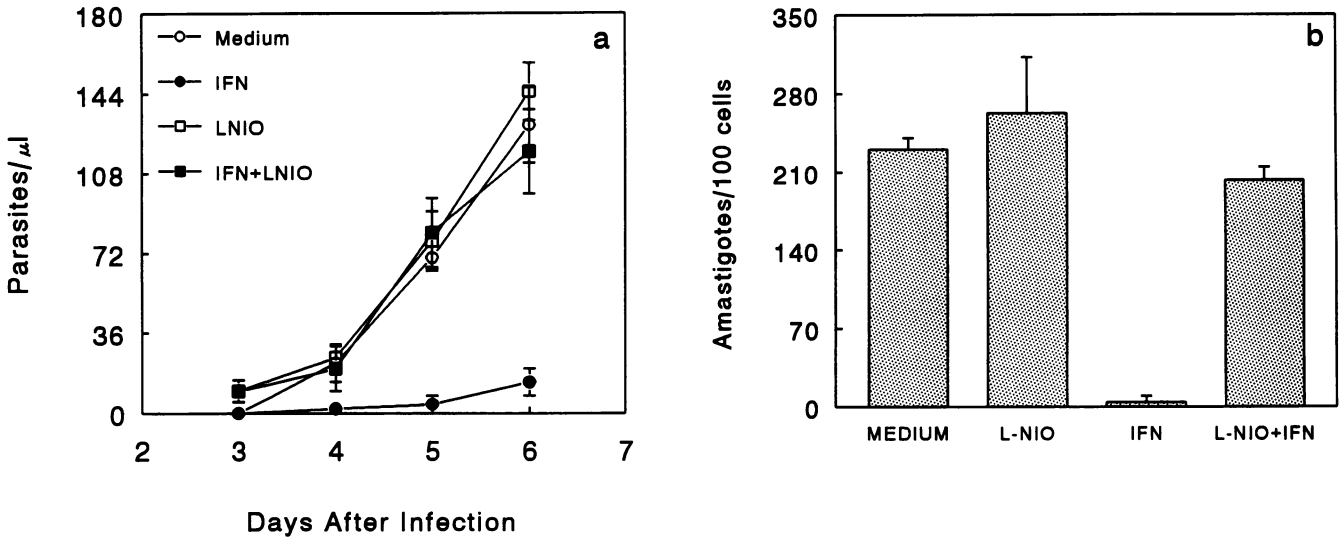

FIG. 3. L-NIO reverses the trypanocidal activity of the IFN-γ-activated macrophages. The infected cells were incubated with RPMI 1640 only (Medium), 100 U of IFN-γ per ml, L-NIO, or IFN-γ plus L-NIO at 37°C in 5% CO_2 for the times indicated. Parasite growth in macrophages was evaluated by counting the trypomastigotes released at the indicated times after infection (a) and by counting intracellular amastigotes 48 h after infection (b). Each point is the mean ± standard error of the mean and is representative of four independent experiments.

observed when the parasites were incubated with SNAP for 24 or 48 h. A concentration of SNAP as low as 15.6 μM was able to reduce the number of viable parasites by 62 and 88% following incubation for 24 and 48 h, respectively (Fig. 4). Penicillamine (control) did not affect the motility of the parasites at any of the doses tested. Parasites, trypomastigotes, or amastigotes incubated for 48 h in the presence of 31.2 to 500 μM SNAP were unable to subsequently infect and grow in normal macrophages over a 72-h period, while those not previously exposed to SNAP were able to do so (data not shown). This model is interesting because it avoids the possible interference of any other mediator. SNAP in solution at 37°C releases only NO and penicillamine (100 μM SNAP gives 1.4 μM NO per min at pH 7). These results suggest a direct effect of NO on *T. cruzi* survival, including that of amastigotes, the form most likely killed by activated macrophages. Other investigators have shown the microbicidal effects of NO donors such as sodium nitroprusside (24) and SNAP (2) on *L. major*. However, our data do not exclude the possibility that NO is also involved in the immunosuppression observed during the acute phase of *T. cruzi* infection in mice (31), as described for *Trypanosoma brucei* (39), *T. gondii* (3), and *Listeria monocytogenes* (18) infections. Possibly, the reactive nitrogen intermediate synthesis inhibition in *T. cruzi*-infected mice results in decreased suppression and, as a consequence, increased inflammatory reaction in tissues. The parasitemia could increase in these treated animals because of the small production of NO. This hypothesis is under investigation.

The susceptibility of BALB/c mice could be explained by the relative inability of macrophages to produce adequate levels of NO due to a reduced induction of NO synthase by IFN-γ and TNF-α (23). However, while C57BL/6 mice are susceptible to the Tulahuen strain (37), they are resistant to the Y strain of *T. cruzi*. Also, we have previously shown that susceptible mice produce interleukin-10 (IL-10) and transforming growth factor β (TGF-β) during acute infection with *T. cruzi* and that these cytokines block the ability of IFN-γ to inhibit the intracellular replication of parasites in mouse peritoneal macrophages (37, 38). The ability of IL-10 and TGF-β to suppress microbicidal activity was positively correlated with inhibition of nitrite generation in macrophage culture supernatants (4, 11). These results imply that IFN-γ-induced macrophage activation during *T. cruzi* infection in vivo is modulated by the levels of TGF-β and IL-10. These cytokines can inhibit NO production by macrophages (4, 11), and IL-10 can regulate IFN-γ production (8). Overall, the levels of NO synthase induction in vivo, which would determine the resistance to a specific infection, may be determined by the balance between the release of

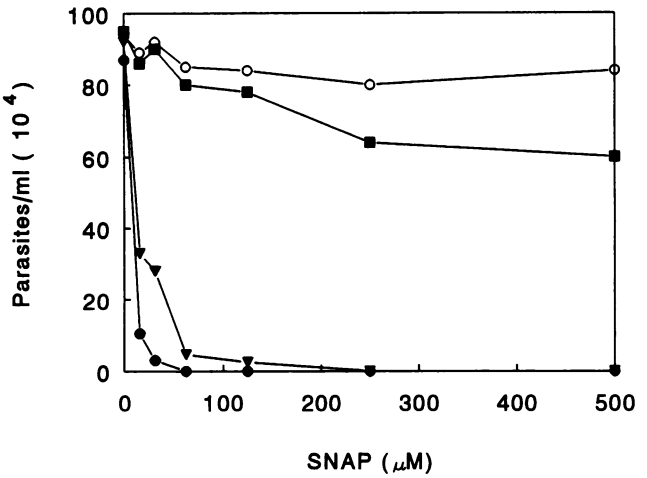

FIG. 4. Dose- and time-dependent killing of *T. cruzi* in vitro by the NO donor SNAP. SNAP (closed symbols) or penicillamine (open circle) at various concentrations was added to the parasites and incubated for 12 (squares), 24 (triangles), or 48 (circles) h. Parasite viability was assessed by determining the number of motile forms in a hemocytometer. Each point is the mean for an experiment performed in triplicate.

cytokines that induce NO synthase and the release of those that inhibit this induction. As we have previously reported (37, 38), susceptible animals produced higher levels of IL-10 and TGF-β than did resistant mice. These results are in accordance with the present data, showing that the levels of NO in plasma and in supernatants of splenocytes from *T. cruzi*-infected resistant mice are higher than those in those from susceptible animals.

This study was supported by grants from FAPESP and FINEP and by fellowships from CAPES (G.N.R.V.) and CNPq (F.Q.C. and J.S.S.).

We thank W. C. R. Silva and J. A. Siqueira for technical assistance.

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